Comparative cryopreservation study of trochophore larvae from two species of bivalves: Pacific oyster (*Crassostrea gigas*) and Blue mussel (*Mytilus galloprovincialis*).

E. Paredes [1*], J. Bellas [2], S.L. Adams [3].

[1] Departamento de Ecoloxia e Bioloxia Animal, Universidad de Vigo, Estrada Colexio Universitario s/n, 36310 Vigo, Galicia, Spain.

[2] Instituto Español de Oceanografía, Centro Oceanográfico de Vigo, Subida a Radio Faro 50, 36390 Vigo, Galicia, Spain.

[3]Cawthron Institute, Private Bag 2, 98 Halifax Street East, Nelson, New Zealand 7010

Corresponding Author:

[*]E. Paredes eparedes@uvigo.es
Universidade de Vigo
Facultade de Ciencias del Mar
Departamento de Ecoloxía e Bioloxía Animal
Carretera Colexio Universitario s/n
C.P. 36310
Vigo (Pontevedra) Spain
Tlf. (+34) 986 81 40 87
Fax (+34) 986 81 25 56

Abstract

Oysters and mussels are among the most farmed species in aquaculture industry around the world. The aim of this study was to test the toxicity of cryoprotective agents to trochophore larvae from two different species of bivalves and develop an improved cryopreservation protocol to ensure greater efficiency in the development of cryopreserved trochophores (14 h old Oyster larvae and 20 h old Mussel larvae) to normal D-larvae for future developments of hatchery spat production. The cryopreservation protocol producing the best results for Oyster trochophores (60.0±6.7% normal D-larvae) was obtained holding at 0ºC for 5 min then cool at 1ºC min^{-1} to -10 ºC and hold for 5 min before cooling at 0.5ºC to -35ºC, hold 5 min and then plunge into liquid nitrogen (LN), using 10% EG. For mussel experiments, no significant differences were found when cooling at 0.5ºC min^{-1} or at 1ºC min^{-1} for CPA combinations with 10% EG and at 0.5ºC min^{-1} c. Using these combinations, around half of trochophores were able to develop to normal D-larvae post-thawing.

Keywords: Cryopreservation, *Crassostrea gigas*, *Mytilus galloprovincialis*, Trochophore larvae.

1. Introduction

Oysters and mussels are among the most farmed species in the aquaculture industry with around 2 million tonnes of mussels farmed and 4.5 million tonnes of oyster produced worldwide in 2010 (FAO 2010). The Pacific oyster, *Crassostrea gigas,* is among the most produced aquaculture species in New Zealand, with more than 230 oyster farms in 2005 producing around 3300 tonnes of oysters (US$20 million) according to FAO in 2010. Spain is also a big European oyster producer (4565 tonnes, data from ICEX 2010) but the most farmed bivalve species in Spain is the mussel, *Mytilus galloprovinciallis*, which constituted 82% of Spain's marine aquaculture products in 2010 (FAO, 2010). Not long ago, the culture of those species, *C. gigas* and *M. galloprovincialis,* relied on the collection of natural seed because is a simple and cheap option, with *C. gigas* in New Zealand as well as in other countries as France or the US, this is no longer an option due to the Herpes-Virus infection. Diseases like Ostereid herpes virus 1 (OsHV-1) which has reduced the worldwide Oyster production - affecting several countries like France, Spain, New Zealand or the US- together with increasing environmental human pressure and weather unpredictability are sources of uncertainty and high economic risk for this economic activity. In response to the economic importance of these two bivalve species, there is an increasing interest in developing hatchery spat production to ensure a reliable, safe supply of on-growing biological healthy material and selective infection-resistant broodstock (Robert *et al*. 1999, Adams *et al*. 2004).

Cryopreservation of shellfish embryos and larvae can improve aquaculture management, enhance breeding design flexibility and provides a reference family for selective breeding. Cryopreservation can enable enough spat supply in hatcheries, without the need to condition broodstock for out of season production. Moreover, cryopreservation techniques may help to manage the biological and commercial risks due to diseases or market changes. Indeed, cryopreservation of gametes is already widely used in many farmed aquatic species. Cryopreservation research has been carried out mainly with Pacific oysters (Chao *et al.* 1997, Lin *et al.* 1999, Tervit *et al.* 2005, Usuki *et al*. 2005, Adams *et al*. 2008), whilst much less effort has been devoted to mussels (Toledo *et al.* 1989, Adams *et al.* 2009, Wang *et al.* 2011).

The cryopreservation of sperm is quite-well defined for most bivalve species (e.g. Adams *et al.* 2004, Di Matteo *et al.* 2009). On the contrary, oocytes and early embryos are more difficult to cryopreserve. The cryopreservation of trochophore larvae can be considered as an alternative to separately cryopreserving oocytes and sperm, since previous studies with the oysters *C. gigas* and *C. rhizophorae*, and with the mussel *M. edulis* have shown that the more advanced the developmental stage is, the more resistant cells are to cryoprotectant toxicity, and the higher the post-thaw survival results (Toledo *et al.* 1989). These studies together with our experiments on Greenshell^{TM} mussel trochophores (Paredes *et al.* 2012) lead us to select the trochophore larval stage for further detailed cryopreservation studies. The aim of this study was to test the toxicity of cryoprotective agents to trochophore larvae from two different species of bivalves and develop an improved cryopreservation protocol to ensure greater efficiency in the development of cryopreserved trochophores to normal D-larvae.

2. Materials and Methods

2.1. Gamete collection and handling

Mature ripe oysters (*Crassostrea gigas*) were obtained from oyster farms in Northland, New Zealand, and maintained during the natural spawning season at the Cawthron Aquaculture Parkin Nelson. Sperm and oocytes were obtained by "strip spawning". The oysters were opened and a small sample of gonad tissue was examined microscopically to determine sex. Oocytes were collected by lacerating the gonad wall with the tip of a pipette and gently scraping and washing the gonad contents into 1 L beakers filled with 1 μm filtered seawater (FSW). Oocytes were maintained at 4°C to minimise any loss of viability associated with aging. Sperm were collected into plastic containers in the same manner, but held undiluted at 4°C prior to experiments. Oyster oocytes and sperm (500 sperm/egg) were mixed during a contact time of approximately 15 to 20 min, before being transferred into tanks containing ~150 L of FSW and 1 mg L^{-1} ethylenediaminetetraacetic acid (EDTA), at a temperature of 22°C and density of 15×10^6 oocytes/tank.

Mature blue mussels (*Mytilus galloprovinciallis*) were obtained from the wild in the south margin of Ria de Vigo (Galicia, NW Spain) and were maintained at the Estación Científica Mariña de Toralla (ECIMAT). Thermal cycling was used to induce mussels to spawn as described by Bellas et al. (2005). Oocytes were examined for maturity

based on their shape and colour, and sperm were visually assessed for vigorous motility after activation with FSW. Mussel sperm and oocytes were allowed a contact time of 15 to 20 min before being transferred into tanks containing 30 L of 1 µm filtered seawater (FSW) and 1 mg L^{-1} ethylenediaminetetraacetic acid (EDTA), at a temperature of 20ºC and a density of 1×10^6 oocytes/tank.

After 14 h for oysters) and 20 h for mussels, trochophores of both species were collected by gently siphoning the contents of the tanks through a 15 µm screen semi-submerged in order to avoid larvae being physically damaged. Trochophores were then gently swirled on the screen, concentrated and placed into 50 mL falcon tubes for experiments. For each experiment, three replicate runs were carried out. Oocytes and sperm were pooled from three individuals per pool and three female pools were used for each experiment.

2.2. Experimental reagents

CPA combinations of ethylene glycol (EG) and trehalose (TRE), obtained from Sigma-Aldrich chemicals (St Louis, MO, USA), were prepared in Milli-Q water. In addition, polyvinylpyrrolidone (PVP) obtained from Sigma-Aldrich was used in oyster experiments. Bovine serum albumin (BSA) was used during thawing (GIBCO Invitrogen and Sigma-Aldrich).

2.3. Toxicity tests

Toxicity trials with mussel and oyster trochophores were undertaken using different concentrations of EG and TRE. In addition, PVP was also tested for toxicity with oyster embryos on the basis of previous experimental results (unpublished data). Cryoprotectant solutions were added 1:1 to a high concentrated suspension of trochophores in one step. Samples were allowed 15 min for equilibration and then were incubated in FSW at a density of 40 embryos mL^{-1}, for a further 24 h at 20-22ºC until D-stage was reached. Larvae were then fixed by adding formalin (1% v/v final concentration).

The percentage of trochophores developing to normal D-larvae was calculated as an indicator of CPA toxicity and the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) were determined. The percentage of D-larvae was calculated in each experiment and at least three replicates were counted

under a light microscope for each treatment. The average count of the three replicates was normalized against the controls before being used in further data analysis.

2.4. Cryopreservation experiments

Following the toxicity evaluation of the CPAs, cryopreservation experiments were carried-out using a Freeze Control System (Cryologic Pty Ltd). Cryoprotective agents were prepared at twice their final concentration (in Milli-Q water) and 1 mL of CPA was then added in a single step to 1 mL of trochophores suspension, allowing for 15 min equilibration before freezing. Trochophores were then loaded into 0.25 mL plastic straws (IMV Technologies, France) and sealed with PVC powder. Two cooling rates were tested 0.5ºC min^{-1} and 1ºC min^{-1} as described: hold at 0ºC for 5 min then cool at 1ºC min^{-1} to -10ºC and hold for 5 min before cooling at either 0.5ºC or 1ºC to -35ºC, hold 5 min and then plunge into liquid nitrogen (LN). Seeding was always checked at the -10 ºC hold and straws were manually seeded using a LN cooled cotton bud where necessary.

Straws were thawed in a 28ºC water bath until the ice had melted. Trochophores were then diluted 1:1 with FSW containing 0.1% (w/v) BSA and left for 15 min before being diluted into 20 L buckets with FSW containing EDTA for a further 24 h incubation (density of ~300,000/bucket) at 20-22ºC, until D-larval stage was attained. D-larvae were collected on a 45 μm mesh screen and samples were fixed with formalin (1% v/v final concentration) for larval length measurement and % normal D-larvae count.

2.5 Effects of cryopreservation on larval development

Finally, a mussel trochophores cryopreservation experiment was performed using 10% EG + 0.2M TRE. Thawed mussel larvae were then incubated for 48, 72 and 96 hours to determine whether the average D-larvae size changed significantly along the first days of incubation due to recovery from exposure to low temperatures.

2.6. Statistical analysis

Statistical analyses of toxicity tests data were conducted using the SPSS version 5.0 statistical software. Differences in the percentage of normal larvae and larval growth among each one of the treatments and control were analyzed by one-way analysis of variance (ANOVA), followed by the Dunnett's test for calculation of the CPA's No

observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC). Cryopreservation data was analyzed using R 2.12.2 free software with the additive main effects model being used for ANOVA-II without replication (pool effect was introduced as another factor into the analysis). In all instances, a P-value of less than 5% was considered significant.

3. Results

3.1. Toxicity tests

The NOEC for oyster trochophores was 15% EG while LOEC was 20% EG. There was no significant difference in toxicity between using EG in combination with TRE alone or with both TRE and PVP, as long as the EG concentration was less than 20% (Figure 1). Two non-toxic levels of EG were selected to continue with cryopreservation experiments using oyster trochophores, 10% EG and 15% EG in combination with TRE and PVP.

The NOEC for mussel trochophores was also 15% EG and the LOEC was 20% EG. Below the LOEC level there was no significant difference in toxicity between using EG alone or in combination with TRE 0.2 M or 0.4 M**.** For mussel trochophores, 10% EG and 15% EG in combination with TRE were selected as non toxic levels of CPAs for further cryopreservation experiments (Figure 2).

3.2. Cryopreservation experiments

Development of thawed oyster trochophores to D-larval stage was highest (60.0±6.7%; mean ± SE) when trochophores were frozen in 10% EG and a cooling rate of 0.5ºC min^{-1} was used (Figure 3). The addition of TRE or TRE plus PVP did not improve D-larval yield from that obtained with EG alone. The size of D-larvae obtained from thawed oyster trochophores was significantly different to the size of unfrozen control larvae (10.3% smaller on average; data not shown).

For mussels, the percentage of D-larvae developing from thawed trochophores was highly variable between the replicate pools evaluated. Despite having taken into account pool variability in our statistical analysis no significant differences were found when cooling at 0.5 ºC min^{-1} or at 1 ºC min^{-1} for CPA combinations with 10% EG and at 0.5 ºC min^{-1} with 15% EG + 0.4M TRE (Figure 4).

Finally, the growth of D-larvae produced from thawed mussel trochophores was examined over a four day incubation period. Whereas unfrozen larvae increased in size over the incubation period, thawed larvae showed no significant increase in size (Figure 5).

4. Discussion

Our test species developmental stage was selected because trochophores had been widely documented to be more resistant to CPA toxicity (Chao *et al.* 1997, Gwo 1995, Nascimiento 2005) than other stages. We also choose to use this stage in our previous work on Greenshell™ mussels (*Perna canaliculus*; Paredes *et al*. 2012).

Cryoprotectant toxicity tests are an important step in optimizing a cryopreservation protocol for a given species or cell type. In this study, the CPA toxicity tests with bivalve trochophores yielded a NOEC of 15% EG and a LOEC of 20% EG for both species. Below this concentration there were no toxic effects of EG, whether it was used alone or in combination with TRE and/or PVP. These results are consistent with our previous studies on Greenshell™ mussel trochophores (Paredes *et al.* 2012). However, the response to CPA exposure of the same developmental stage in different species should not be assumed since CPA tolerance can be specific to a given species and/or cell type/developmental stage and toxicity studies may or may not take into account the osmotic tolerance limits and permeability of the particular cell that is of interest (Nascimiento *et al*. 2005) (Chao *et al.* 1997, Gwo 1995, Paniagua 2001, Tervit *et al*. 2005). Here, cryoprotectants were added in a single step and it is possible that cells could tolerate a higher cryoprotectant concentration if stepwise methods of addition and removal had been used. The variability among responses to CPAs found in literature confirm that different species and different developmental stages may behave in a different way when exposed to a CPA and cryopreserved, although our results also suggest that the same development stage of similar species might behave in a similar way due to shared characteristics on membranes composition, cell structure and permeability as well as similar response to CPAs toxicity (Paredes *et al.* 2012)

In the present study, the presence of TRE and/or PVP did not reduce or enhance EG toxicity below a concentration of 20% EG. Results of our cryopreservation experiments

showed that 10% EG alone yielded the best results for oyster trochophores with 60±6% developing to normal D-larvae when a cooling rate of 0.5ºC min^{-1} was used.

The highest % normal D-larvae (over 80%) were achieved with one single pool, using 10 % EG + 0.2 M TRE, and a cooling rate of 0.5ºC min^{-1}. In this case, considering this high outcome as an outlayer and examining the results achieved for the other two pools and other treatments with no significant differences, namely 10% EG + 0.2 M TRE and 10% EG +0.4 M TRE (1ºC min^{-1}), and 15% EG + 0.4 M TRE (0.5ºC min^{-1}), with average percentages of normal D-larvae of 48.9±7.6%, 47.5±11.2% and 48.8±17.5%, respectively, we obtained more probable consistent, results. Concluding, our protocol for mussel cryopreservation provides an outcome around 50% normal larvae.

As for oyster larvae, mussel D-larvae that were produced from thawed trochophores were found to be significantly smaller (17.9% smaller) than controls, and these size differences persisted over the 4 day incubation that with cryopreserved larvae showing no growth over this time. These results are consistent with Paredes *et al.* (2012), where observed size differences between frozen and fresh Greenshell™ mussel D-larvae that were maintained till settlement and with Wang *et al*. (2011) with *Mytilus galloprovincialis* where cryopreserved D-larvae remained smaller than controls during the first week of incubation following cryopreservation. However the incubation time was too short to derive any conclusions about the viability of the resulting larvae.

For both, oysters and mussels, results were very similar while freezing at 0.5 ºC min^{-1} or 1 ºC min^{-1} with 10% EG, and no significant differences among cooling temperatures were found. However, when using 15% EG, better results were obtained at 0.5ºCmin^{-1}. Survival of cryopreserved and thawed bivalve trochophores was 60.0±6.7% for oysters and 48.9±7.6% for mussels.

In conclusion, a successful freezing protocol for both bivalve species trochophore larvae (*M. galloprovincialis* and *C. gigas*) was developed with specific adaptations for the different bivalve requirements. Further investigations to increase the percentage of normal D-larvae resulting from cryopreservation, and cryopreserved larval growth and health along incubation to spat, would ensure the future of application of

cryopreservation techniques as useful tools to guarantee a reliable supply of on-growing biological material in developing hatchery spat production.

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Figure captions

Figure 1. - Percentage of D-larvae developing from oyster trochophores following exposure to increasing concentrations of EG in combination to TRE (M) and/or 1% PVP. The FSW bar represents a handling control. All data has been normalized to the controls. Mean ± Standard Error (SE).

Figure 2. - Percentage of D-larvae developed from Mussel trochophores following exposure to increasing concentrations of EG in combination to TRE (M). All data has been normalized to the controls. Mean ± Standard Error (SE).

Figure 3.- Percentage of Pacific oyster trochophores developing to D-larvae following cryopreservation at 0.5 ºC min^{-1} and 1 ºC min^{-1} in 10 or 15% ethylene glycol (EG) alone or in combination with 0.2 M trehalose (TRE) with or without 1% polyvinylpyrrolidone (PVP). All data has been normalized to the controls. Mean ± Standard Error (SE).

Figure 4.- Percentage of Mussel D-larvae following cryopreservation at 0.5 ºC min^{-1} and 1 ºC min^{-1} in 10 and 15% EG in combination to TRE(M). All data has been normalized to the controls. Mean ± Standard Error (SE).

Figure 5. - D larvae size for unfrozen controls and frozen trochophores at 48, 72 and 96 incubation hours post fertilization. Mean ± Standard Error (SE).

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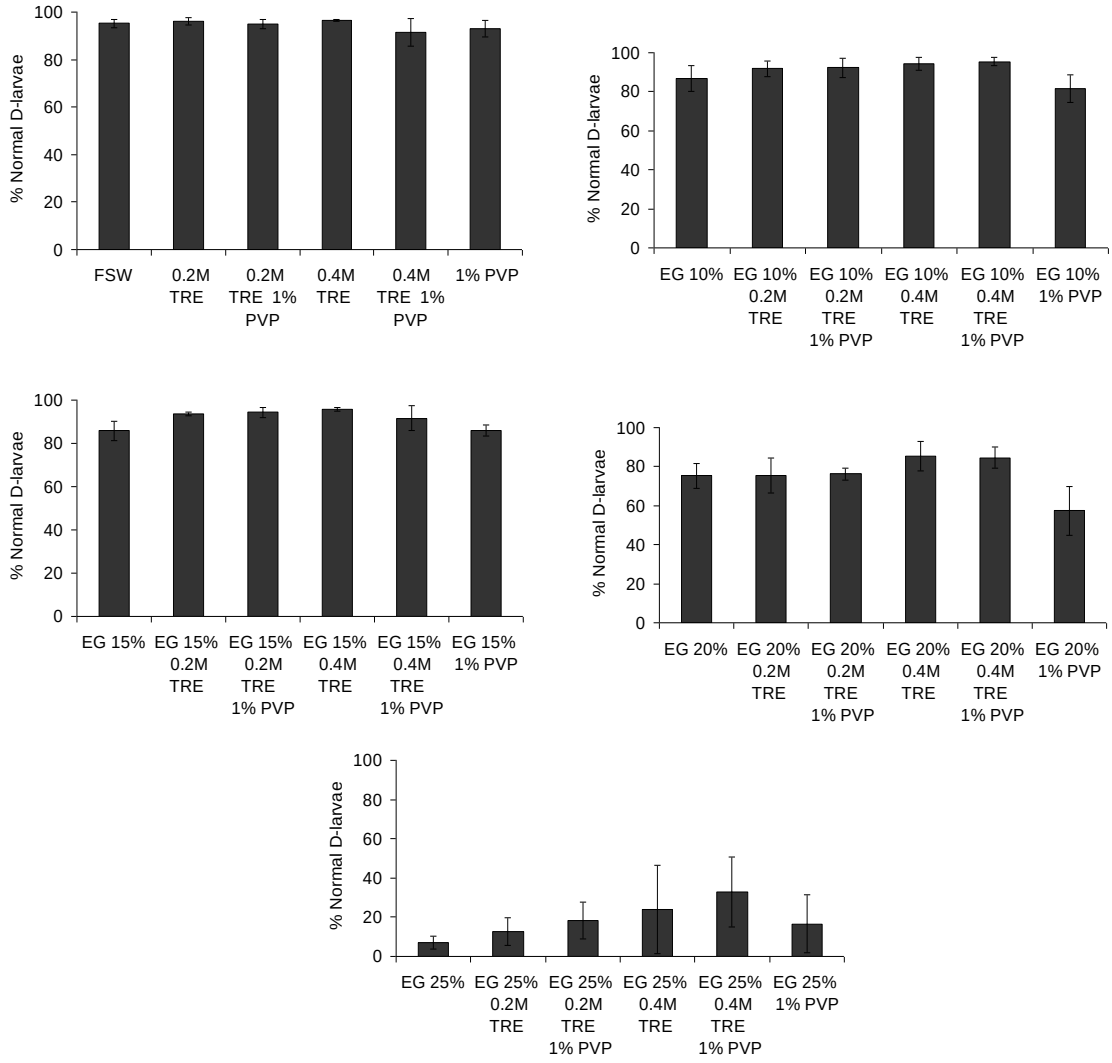

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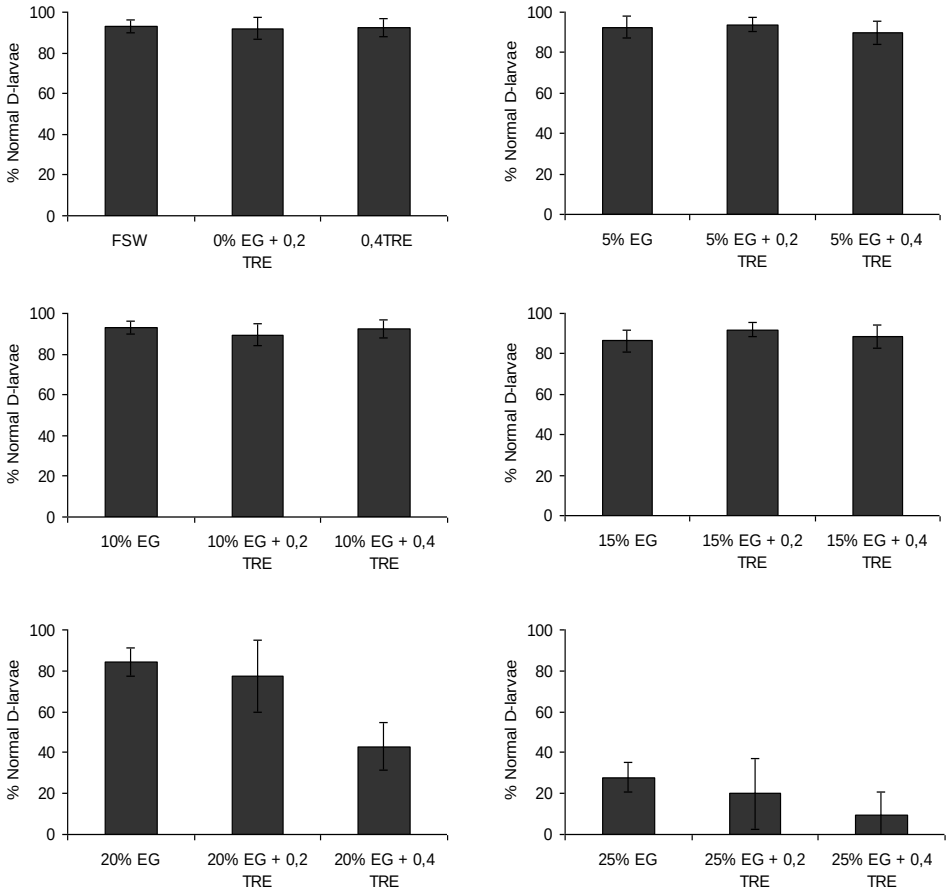

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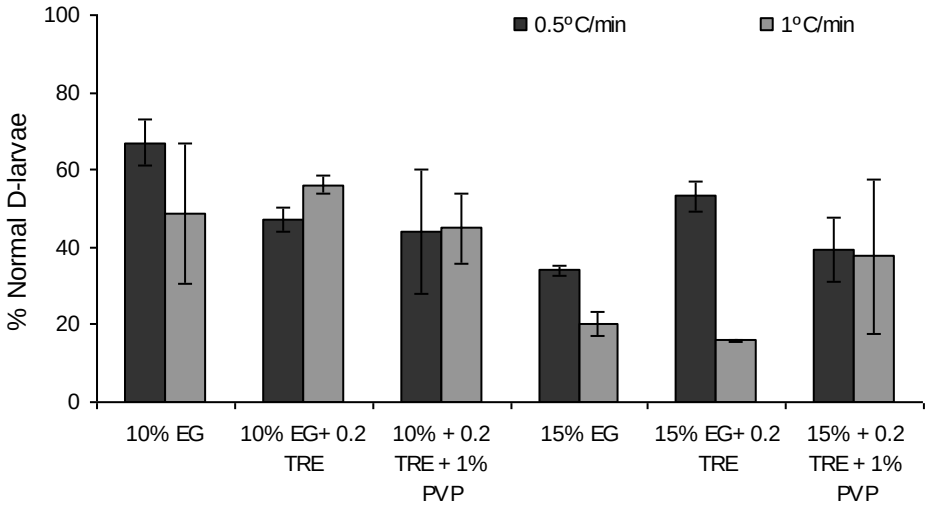

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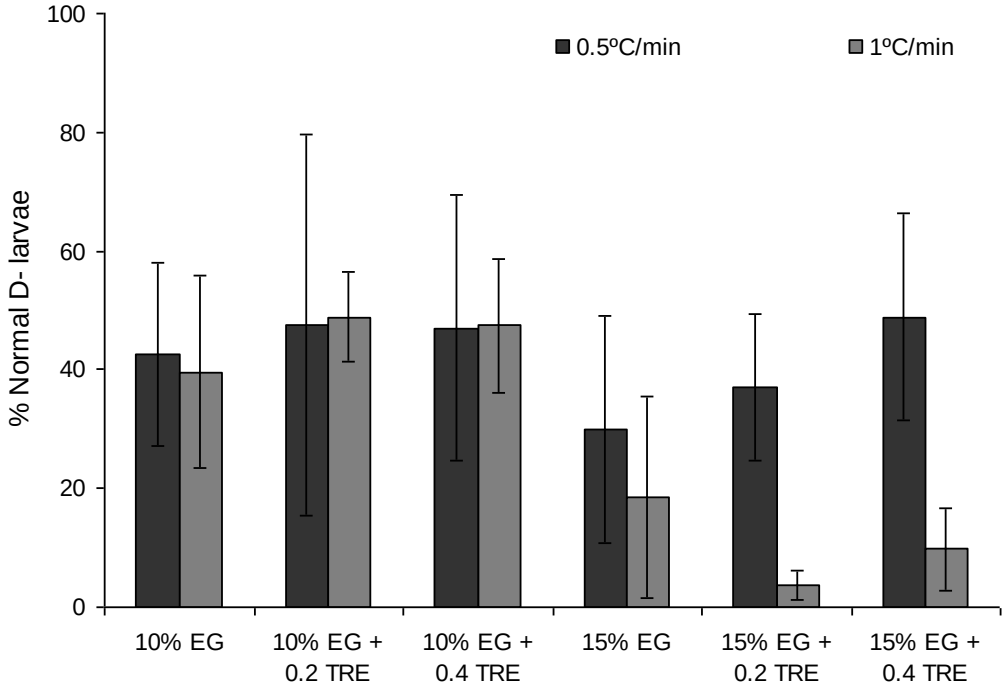

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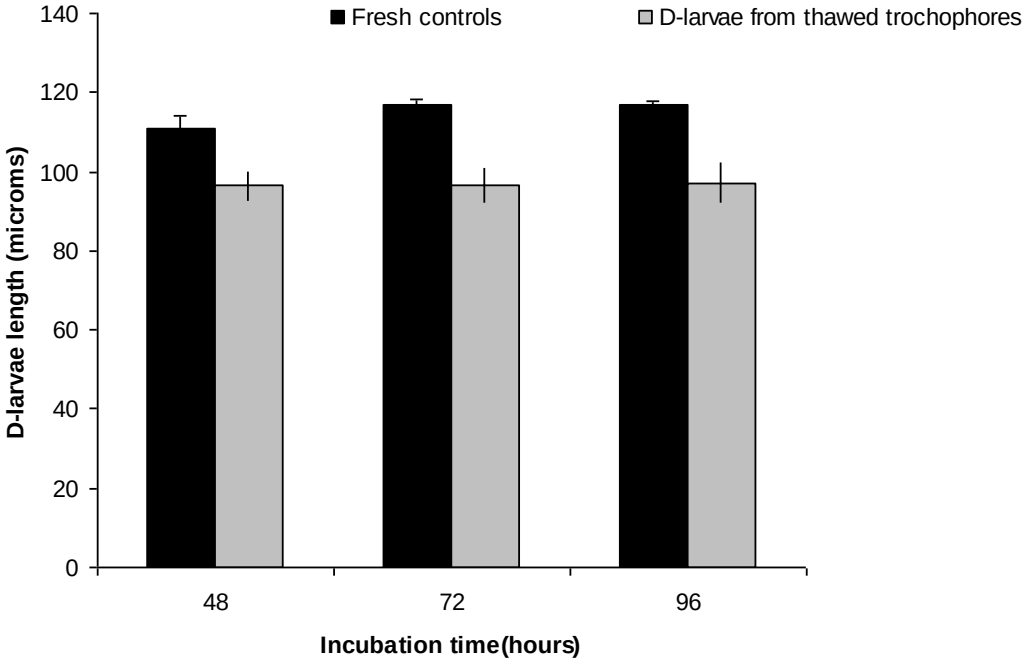

Figure 5